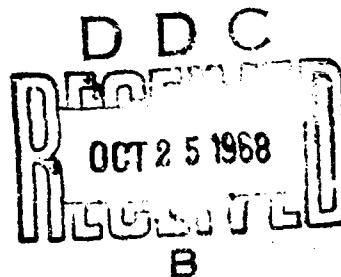


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SURVIVAL OF COLONY-FORMING UNITS AND SURVIVAL  
OF IRRADIATED MICE TREATED WITH AET OR ENDOTOXIN

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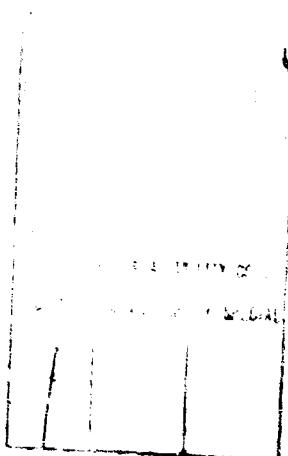
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## ABSTRACT

When mice are given radiation exposures producing the hematopoietic syndrome, it is assumed that it is the killing of hematopoietic stem cells and the leuko- and thrombocytopenias which ultimately develop that predispose the animals to infection, hemorrhage and death. The colony-forming unit (CFU) has many attributes of a (the) hematopoietic stem cell, and it might be expected that a high correlation should exist between CFU survival and survival of the animal. Some earlier studies have supported this correlation, whereas, others have not. In the present experiments three methods of CFU enumeration (endogenous, exogenous, and donor) have been used to evaluate this correlation in mice "protected" with AET or bacterial endotoxin. The results show that the different CFU enumeration procedures yield somewhat different results, yet under certain conditions the  $LD_{50}$ 's for AET- or endotoxin-treated mice may be predicted within 5 - 10% from CFU survival curves. In spite of the good correlation between CFU survival and probability of survival of the mouse, it is proposed that the CFU is not the stem cell which determines the radiation sensitivity of the mouse.

## SUMMARY

### Problem:

The survival of hematopoietic stem cells is a major determinant of survival in middlethally irradiated animals. By knowing the relative number of stem cells in the mouse, the radiation sensitivity ( $D_{37}$ ) of stem cells, and the critical number or fraction of stem cells which is essential for survival of the animal, it should be possible to predict the  $LD_{50}$ . The colony-forming unit (CFU) has many attributes of the hematopoietic stem cell, and the present experiments were designed to evaluate the correlation between CFU survival and survival in mice "protected" with AET or bacterial endotoxin.

### Findings:

Under certain conditions the  $LD_{50}$  for AET- or endotoxin-treated mice may be predicted within 5 - 10% from CFU survival curves. Different methods of CFU enumeration yield somewhat different estimates of CFU survival, and the protective agents change the size distribution of spleen nodules. In spite of the correlation between CFU survival and  $LD_{50}$  demonstrated here, it is proposed that the CFU is not the stem cell which determines the  $LD_{50}$  of mice.

## INTRODUCTION

The colony-forming unit (CFU) exhibits many of the properties which are attributed to the hematopoietic stem cell(s) (1 - 6). Various methods of quantitating radiation responses of CFU's have provided the tool by which the lethality response of animals and/or radiation damage to the hematopoietic system may be interpreted in terms of survival curves derived experimentally for stem cells in the bone marrow or spleen (7 - 9). The conceptual basis for experimental studies relating  $LD_{50}$  and CFU's is the hypothesis that, at radiation exposures producing the bone marrow syndrome, a direct correlation exists between stem cell survival and survival of the animal. In the present experiments we have attempted to evaluate this hypothesis.

Studies of the correlation between radiation responses of CFU's and radiation sensitivity ( $LD_{50}$ ) of animals have one common feature in experimental design, yet three different lines of investigation have been followed. The common feature is that the  $LD_{50}$  of the animal is varied by one means or another, and the CFU responses, in terms of numbers surviving or  $D_{37}$ 's, are measured to determine if the CFU response varies in the same fashion as does the survival response of the animal, viz., an increase in  $LD_{50}$  is accompanied by an increase in the surviving number or the  $D_{37}$ . The different lines of investigation

relate to the choice of means of altering the  $LD_{50}$ . The means used heretofore have been: 1) age-dependent changes involving weanling and adult mice (10 - 13); 2) changes in  $LD_{50}$  produced by radio-protective procedures (14 - 23); and 3) changes in  $LD_{50}$  which are related to recovery from radiation injury (24).

Certain data support the proposed correlation between CFU's and the animals' radiosensitivity, but other data do not. The use of different methods of CFU quantitation as well as details of the experimental designs could contribute in part to contradictory results. The radiation-protection studies with MEG, AET, cystamine and hypoxia differ in some details, but in general they show a correlation between increased survival of mice and survival of CFU's (16, 20 - 23). However, if endotoxin or colchicine are used to increase survival, the relationship is equivocal in the sense that the injection time producing the maximal effects on survival is correlated with the greatest changes in CFU's, but very large increases in CFU's occur under circumstances which have little or no effect on survival (14, 15, 17 - 19). Both endotoxin and AET increase survival of irradiated mice, but different mechanisms are operative (23). Using these substances, we have compared the survival-promoting effects and their influences on recovery from radiation injury (26, 27). In the present experiments this comparison has been extended to their effects on survival of CFU's. In our earlier experiments with endotoxin the results obtained from studies of endogenous or transplantation methods differed somewhat, and we expressed concern about

various factors which could influence the endogenous method of CFU enumeration (19). Therefore, in the present studies of CFU responses and mouse LD<sub>50</sub>, we have compared different methods of quantitating survival of CFU's. The present results show that CFU responses evaluated by different methods do in fact differ in some particulars, yet under certain conditions it is possible to predict the measured LD<sub>50</sub> within 3 - 10% from the survival responses of CFU's.

#### METHODS AND MATERIALS

##### Experimental Animals:

The mice used were LAF<sub>1</sub> female or males 90 - 130 days old which were bred and raised in this Laboratory. They were maintained 10 mice per cage and allowed Purina Laboratory Chow and acidified water ad libitum.

##### Irradiation Procedure:

Mice were exposed to 250 kvp X-rays while restrained in corked, perforated, lusteroid tubes placed on a rotating turntable at 100 cm from the target. The filtration was 0.5 mm of copper and 1.0 mm of aluminum; the half value layer was 1.35 mm of copper. The dose rate at this distance was 23.0 R/min.

##### Protective Procedures: AET and Endotoxin:

Aminoethylisothiouronium bromide hydrobromide (AET), buffered to pH 7.2, was administered intraperitoneally at a dose of 275 mg/kg; this

dose was essentially sublethal, for only an occasional animal died following injection. When AET was given before irradiation, the interval between injection and irradiation did not exceed 15 minutes. In some experiments the same dose of AET was given between 5 and 15 minutes after irradiation.

The endotoxin used in these studies was PIROMEN, a highly purified lipopolysaccharide derived from *Pseudomonas* (28). The stock concentration supplied by Flint Eaton and Company, was 1000  $\mu$ g/ml. The mice were injected intraperitoneally with 0.05 ml (50  $\mu$ g) of the stock solution. This dose produced occasional signs of acute toxicity but was sublethal. All animals were injected 24  $\pm$  1 hours before irradiation.

Femoral Marrow and Spleen Cell Suspensions:

The femurs were excised from a minimum of five decapitated, exsanguinated mice, each taken from a separate cage. A small opening was teased in the intercondylar fossa with a 25-gauge, 1/4" needle mounted on a 1.0 cc syringe. The needle was then inserted into the marrow cavity at the opposite end of the femur between the greater trochanter and the head. The marrow was discharged into a 100 ml teflon beaker by forcing a small quantity of a chilled, balanced salt solution (Hanks) through the marrow cavity. After the femur had been flushed several times, it was placed into a 15 ml bottle containing a small quantity of Hanks' solution. When marrow had been collected from all the femurs, the teflon beaker was swirled to disperse the marrow cells. The marrow suspension was

filtered through fine mesh nylon (pore size of 0.3 mm) into a graduated cylinder. The nylon filter was removed, turned inside-out, and placed in the bottle with the femurs. The bottle was capped and gently agitated to suspend the cells adhering to the filter or the epiphyses of the bones. Marrow cells eluted in this way from the filter or bones were transferred to the graduated cylinder through a clean nylon micro-filter. The latter operation was continued until the desired volume was obtained. During the entire procedure the marrow cells were kept at between 4 - 10° C by performing the various steps within the confines of an open refrigerator. This prevented coagulation. The nucleated cell count was determined as described below.

The spleen was excised and placed on a gauze pad moistened with Hanks solution. The capsule was opened, and the spleen was deposited in the reservoir of a stainless steel filter assembly fitted with a nylon filter with pore sizes of 40 microns. The cells were carefully worked loose from their capsules and washed through the filter into a 40 ml round bottom centrifuge tube. The dissociated spleen cells were centrifuged at 1000 rpm for 10 minutes at room temperature. The supernatant was withdrawn and the spleen cells were resuspended in chilled (4 - 10° C) Hanks solution to the desired volume. The total nucleated cell count was determined with the aid of a hemacytometer using a .06 HCl diluting fluid.

Methods for Enumerating Colony-Forming Units:

Endogenous: The method used is that which was first described by McCulloch and Till in 1964 ( 3 ). In brief, mice which are exposed to approximately 60 - 85% of their  $LD_{50/30}$  show nodules in their spleens when examined 8 days later. The number of spleen nodules is inversely related to the radiation exposure.

Exogenous or Recipient: Recipient mice are subjected to two graded exposures, separated by approximately 2 hours, which total 900 R; a first or primary ( $D_1$ ) exposure is given before the injection of the cell suspension containing CFU's, and a second ( $D_2$ ) or test exposure is given to the transplanted cells. With this method exposure-response relationships are established by comparing the number of CFU's in a nonirradiated suspension to the number of CFU's in that suspension which survive various radiation exposures. Numbers of CFU's were injected, which, according to calculation would result in 8 - 16 nodules/spleen after any test exposure to radiation. This was usually achieved and counts of less than 5 nodules/spleen were rarely encountered. In these experiments male mice were used as donors of marrow or spleen cells and females were used as recipients. When AET was used in connection with this method, the drug was given within 15 minutes before the test exposure.

Donor: This method also involves transplantation, but it is distinct from the recipient method in that the donor mice rather than the recipients

are given the test exposure to radiation. Donor animals, treated or untreated, are given graded test exposures, sacrificed within 30 minutes, and suspensions of their femoral bone marrow or spleen cells are injected into recipients exposed to 900 R. The dilutions used were adjusted, according to the radiation exposure and expected survival, to produce 8 - 16 nodules/spleen.

Counting Criteria for Spleen Nodules:

In view of the abundance of small nodules, especially with the endogenous method, and in view of differences reported earlier in size distribution (19), the question arose as to the effect of size criteria used for enumeration on the exposure response relationship. Therefore, it was deemed desirable to actually size all spleen nodules so that the data could be treated on an expanded basis.

The spleens of the recipient mice were all harvested at 8 days, fixed in alcohol, acetic acid and formalin (AAF), and the nodules were sized with the aid of a stereozoom-dissecting microscope fitted with an optical micrometer. The long axis of all discernible nodules was measured to the nearest 0.1 millimeters.

Statistical Analysis:

Median lethal doses ( $LD_{50}$ 's) and other statistics were computed from a maximum likelihood solution of the regression of the normal equivalent deviate (Probit-5) of percentage of mortality on the radiation exposure in roentgens (R). The regressions were calculated using a

USNRDL program based on probit analysis (29) as adapted to a computer by Aitchison and Brown (30).

Survival curves for spleen nodules were fitted by a least squares regression of the logarithm of the number of nodules on the radiation exposure in roentgens (R). The  $D_{37}$ 's, intercepts, and 95% confidence intervals were derived from the slope and standard error of the fitted curves.

#### EXPERIMENTAL

##### LD<sub>50/30</sub> Determinations:

The effects of AET or endotoxin on 30-day survival of irradiated mice are summarized in Table I. The LD<sub>50</sub> for nontreated control mice was 721 R. When AET (275 mg/Kg) was administered approximately 15 minutes before irradiation, the LD<sub>50</sub> was 1313 R, an increase of 82% over the LD<sub>50</sub> for controls. Injection of AET 15 minutes after irradiation produced no significant increase in the LD<sub>50</sub>, which was 745 R. A 50  $\mu$ g injection of endotoxin given 24 hours before irradiation raised the LD<sub>50</sub> to 919 R, an increase of 27%. It should be noted that the slopes of the exposure-response curve for the control and treated groups did not differ significantly.

##### "Endogenous" Spleen Nodules:

Figure 1 summarizes the results of experiments which relate radiation exposure to number of endogenous spleen nodules in four groups of

animals: 1) untreated controls, 2) mice given AET 15 minutes before irradiation, 3) mice given AET 15 minutes after irradiation, and 4) mice given endotoxin 24 hours before irradiation. The data are presented in terms of nodule counts based on two size categories, 0.5 mm and above<sup>a</sup>, which excludes the background of tiny nodules and restricts the scoring to the large discrete nodules which may be counted easily (Figure 2A), and 0.1 mm and above, which represents the total count; this includes the tiny nodules which are very difficult to count (Figure 2B).

The point to be made from Figure 1 is that neither the protective procedures (AET or endotoxin) nor the size criterion used for scoring produced significant alterations in the slopes of the survival curves. The variations in dose-responsiveness were such that the  $D_{37}$  estimates under certain circumstances were only reliable within a factor of  $\sim 2$ . This occurred in both nontreated controls as well as in treated animals. Even if this high degree of variability were disregarded and the  $D_{37}$ 's were accepted as such, the expected increase in  $D_{37}$  with AET given before irradiation are not observed.

Although the slopes of the survival curves are not changed significantly by AET or endotoxin given before irradiation, the numbers of nodules which occur in the spleen are markedly increased over the control values at any given radiation exposure. On the basis of the fitted curves, the

<sup>a</sup> the largest nodules do not exceed 2.0 mm.

relative increases and controls range from a factor of 10 to > 100. Thus, there is no doubt that protective procedures which increase survival of the mice in some fashion produce an increase in the number of nodules in the spleen. One may attempt to interrelate the numbers of spleen nodules and survival or  $LD_{50}$  of the mouse using the completely empirical procedure described below. In doing this, we have disregarded the variability in endogenous nodule responses and have used the computed  $D_{37}$ 's. The results are rather surprising.

The "survival curves" in Figures 1A and 1B have been extrapolated to the higher exposure ranges in which only fractions of a nodule/spleen would be expected. This was done in order to compare the expected number of nodules present at the measured  $LD_{50}$ 's. The measured  $LD_{50}$ 's for the various protective procedures are plotted on the extrapolated portions of the nodule survival curves. This intercept of the nodule curve and the  $LD_{50}$  exposure is an estimate for the number of spleen nodules present at the measured  $LD_{50}$ . Figure 1A shows that at the measured  $LD_{50}$  of 721 R in control animals, the spleen should contain approximately 0.09 nodules. If 0.09 nodules/spleen is selected as the value at which control mice have a 50% probability of survival, it might be expected that mice given protectant would also have a 50% probability of survival at 0.09 nodules/spleen, irrespective of the absolute exposure in R or the slope of the curve which results in this number of "surviving" nodules. One may in this way obtain a "predicted"  $LD_{50}$  for AET or endotoxin

by merely moving across the X axis and determining from the various nodule survival curves the point on the X axis (the exposure in R) at which the survival curve reaches  $\sim 0.09$  nodules/spleen. The similarity between the measured and the predicted  $LD_{50}$ 's thus indicates the degree of reliability with which the mouse's  $LD_{50}$  may be predicted from the spleen nodule responses. These relationships are summarized in Table XI. The expected  $LD_{50}$ 's are shown separately based on counts of large nodules (from Figure 1A) and based on total nodule counts (from Figure 1B). Based on the counts of large nodules in animals given AET 15 minutes before irradiation, the  $LD_{50}$  was underestimated by approximately 60 R or only about 5%. In the groups given AET after irradiation or endotoxin 24 hours before irradiation the counts of large nodules overestimated the  $LD_{50}$ 's by approximately 21%. On the other hand, total nodule counts came much closer to predicting the  $LD_{50}$  in mice given either endotoxin or AET after irradiation. The estimated  $LD_{50}$ 's were within 9% and 5%, respectively, of the measured  $LD_{50}$ 's. Thus, the interesting point is that in spite of the variations in endogenous nodule responses and the fact that small errors are amplified by the extrapolation procedure used here, the  $LD_{50}$ 's may be predicted within 10%.

Differences in the size distribution of nodules in the various groups (Figure 2) as well as counting errors and inherent variability of the system could partially explain the influence of size criterion on the "accuracy" of  $LD_{50}$  prediction. Figure 2 shows that in control mice

approximately 46% of the nodules were 0.4 mm or less, but in the endotoxin group only approximately 25% of the nodules were in that size category. Therefore, when large nodules are scored, a greater percentage of the total distribution is measured in the endotoxin group, approximately 75%, than in the control group, approximately 54%. The nodules simply tend to be larger in the endotoxin-treated animals than in the controls, and this could contribute to "overestimation" of the  $LD_{50}$  by virtue of the presence of "too many" nodules. When total counts are used, the importance of this size difference is diminished, and the  $LD_{50}$  for endotoxin-treated animals is overestimated to a lesser extent. Since the administration of AET 15 minutes before irradiation also increased the size distribution, one might expect the relationship between nodule size criterion and accuracy of  $LD_{50}$  prediction to vary in the same general fashion as for endotoxin. This is not the case. In spite of the tendency toward larger nodules, the  $LD_{50}$  for AET-treated animals was "underestimated" rather than overestimated using either size criterion, and the total nodule count had less predictive value than the count of large nodules. In the case of AET administration after irradiation, a procedure which increases the occurrence of spleen nodules without increasing the  $LD_{50}$ , the "overestimation" of the  $LD_{50}$  based on large nodules is related to an apparent flattening of the slope of the exposure-response curve as compared with the controls (see Figure 1A).

Donor Experiments:

Another method used to evaluate the radiation responses of colony-forming units and to attempt to compare CFU responses with the mouse's radiosensitivity involved irradiating the animals which served as donors of bone marrow and/or spleen cells. The donor animals were given graded radiation exposures, with or without AET having been given approximately 15 minutes before irradiation. The surviving number of CFU's was determined by sacrificing the animals within one hour after irradiation and injecting the appropriate dilutions of marrow or spleen cells into recipient animals exposed to 900 R. Before dealing with survival of CFU's, it was first necessary to determine if the protective procedures changed the number of CFU's present in the femur or spleen at the time of irradiation.

The content of CFU's in the femoral marrow and spleen of nontreated, nonirradiated mice is summarized in Table III. Also presented are data from "donor mice" which were injected either with AET 15 minutes before sacrifice or with endotoxin 24 hours before sacrifice. The data show that within 15 minutes of injection, AET effected no significant changes in the femur or spleen content of CFU's or nucleated cells. In contrast, mice given endotoxin 24 hours earlier showed of the order of a two-fold increase in spleen content of CFU's; the nucleated cell content of the spleen and the ratio of CFU's/ $10^5$  nucleated cells were also increased. The femur CFU content was not increased, but a significant drop (40%)

occurred in the nucleated cell count; this resulted in approximately a two-fold increase in the ratio of CFU's/ $10^5$  nucleated cells. An estimate of the effect of AET or endotoxin on the distribution of spleen nodule sizes can be extracted from Table III by dividing the femur or spleen content based on counts of large nodules (0.5 mm+) by the content based on total nodules counts, viz., in the femurs and spleens of controls; the fraction of large was  $9.2/12.7 = .72$  and  $1.8/2.7 = .67$ , respectively. Since in the treated groups the maximum difference from control values was within 7%, we will assume that the size distributions of nodules in the treated and control animals were similar.

The numbers<sup>a</sup> of CFU's surviving graded radiation exposures in the femur or spleen of AET-treated and control mice are shown in Table IV. These exposure-response relationships are presented in terms of the two nodule size criteria, 0.1+ and 0.5+, and the survival curves in Figure 3 were computed from the mean CFU counts based on the 0.5+ size criterion. In Figure 3 the survival curves were fitted to points at 150 R and above in the controls and to points at 283 R and above in the AET-treated animals; this was done to avoid the shoulder region and to restrict the curve fitting to the exponential portion of the survival curve. However, it is somewhat difficult to establish what is, in fact, the exponential

<sup>a</sup> These values do not represent the total CFU content of the femur. No adjustment has been made for injected CFU's which do not localize in the spleen.

portion, especially in the case of the AET-treated animals. In general, the CFU survival data from the treated animals are more variable than in the controls, and the observed points are less well fitted by the survival curves shown in Figure 3. This was particularly the case at exposures of < 400 R. On the other hand, it is quite clear that one has several options in the curve fitting, and the inclusion of more points, e.g., 192 and 240 R from Table IV, could also yield a survival curve which is an acceptable fit to the data. This matter of curve fitting assumes importance in connection with the inferences to be drawn from the data in terms of the estimation of AET-protection ratios and prediction of the  $LD_{50}$ .

The dependence of  $D_{37}$ , intercept value and a parameter of goodness of fit, the correlation coefficient, on the size criterion and the assumptions concerning the portion of the curve to be fitted are summarized in Table V. The points to be made from these data are: 1) in the controls the  $D_{37}$ 's were essentially unaffected by either the size criterion or the assumptions pertaining to shoulder width (fitting points of 120+ or 150+); 2) in the AET group the size criterion had no effect on the  $D_{37}$ , but fitting the survival curve to points at 283 R+ rather than 194 R+ decreased the  $D_{37}$  by 25 - 30 R. This small decrease in  $D_{37}$  is not significant, but it makes a rather large difference in the AET-protection ratio, the estimate of the surviving number of CFU's at the  $LD_{50}$ , and the predicted  $LD_{50}$ . For example, the protection ratios for femur and spleen CFU's are ~ 1.1 or 2.0 or 1.1 or 2.2, respectively, depending upon the curve fitting and the assumptions made concerning the width of the shoulder of the

survival curve (Table VI). In spite of this, the results in Table VI show that by extrapolation the  $LD_{50}$  for the AET-treated mouse may be predicted within 12%.

The size distributions of the nodules which were counted in the donor experiment are shown in Figure 4. AET did not markedly influence the size distribution of either femoral or splenic CFU's. This figure also shows a small difference in the size distribution of splenic and femoral CFU's. In the spleen 60% of the nodules were large, whereas, the value for the femur was 70%.

#### Recipient Experiments: Exogenous CFU

The third method used to study radiation effects on CFU's involves the experimental design which was described by Till and McCulloch in their initial contribution in this area (1). It is noteworthy that the variability of this method is much less than with the endogenous or donor methods; this fact is illustrated in Figures 5 - 9 by the comparatively small confidence intervals of the  $D_{37}$ 's. In this method the normal bone marrow or spleen cells to be tested are injected into irradiated recipient mice. The test cells are irradiated in vivo by giving the recipient animal graded radiation exposures. When protectants like AET are involved, the agent is given to the recipient animal at the appropriate time, viz., 15 minutes, before irradiation.

The radiation response of normal bone marrow evaluated by this method is shown in Figure 5. All spleen nodules were sized and the responses are presented in terms of counts of large nodules (Figure 5A) and in terms of

total nodule counts (Figure 5B). The criterion of size distribution did not materially alter either parameter of radiation response, namely the  $D_{37}$  or the extrapolation number. Fitting the curves to points above 74 or 149 R also had little effect. The  $D_{37}$ 's were ~80 R, and the extrapolation numbers ~1.7 - 2.2.

Using this same method, the radiation response of spleen cells was evaluated and the data are summarized in Figure 6. Based on large and total nodule counts, the curves fitted to all observed points gave  $D_{37}$ 's of 71 and 73 R, respectively, and the extrapolation numbers were 1.27 and 1.01. Eliminating the two data points at < 75 R from the regression did not change either the  $D_{37}$  or the extrapolation number. Eliminating the points below 150 R yielded a  $D_{37}$  of 37 R (63 - 145) with an extrapolation number of 0.30 based on large nodules, and a  $D_{37}$  of 84 R (61 - 135) and an extrapolation number of 0.01 based on total counts. It should be noted that in the exposure range above 149 R considerable scatter was observed.

Injecting AET into the recipient animals before the test cells were irradiated markedly increased the  $D_{37}$  for CFU's obtained from the femur and spleen. Figure 7 summarizes the results with CFU's from bone marrow, with the two size criteria being plotted separately in Figures 7A and 7B. The curves are based on exclusion of points below 75 R for controls (Figure 5), and below 150 R for AET. Again, the size criterion had a negligible effect on the  $D_{37}$  and extrapolation number. The extrapolation numbers of the AET and control curves were quite similar. The AET-

protection ratio was 1.74 based on either large or total nodule counts.

The size distribution of spleen nodules resulting from the transplantation of marrow from AET-treated or control mice was similar; the large nodule category comprised 71% and 68%, respectively, of the total distributions.

The results with transplanted spleen cells (Figure 8) show that AET produced a large increase in the  $D_{37}$ 's, but the matter of common intercepts for the control and AET-survival curves was somewhat less clear. The CFU-survival curves from the AET-treated animals had the interesting property that, if the two points below 150 R are excluded from the curve fitting, the Y intercepts were slightly increased; a comparable increase was not observed in control animals when the shoulder area was excluded from the curve fitting (Figure 7). Therefore, AET may, in this fashion, affect the shoulder area of the CFU-survival curves derived from the transplanted spleen cells. Based on counts of large nodules, AET increases the  $D_{37}$  by a factor of 1.75 - 1.87<sup>a</sup> and on the basis of total nodule counts by a factor of 1.62 - 1.72<sup>a</sup>. The nodule size distributions were similar; 72% large nodules in the AET group and 69% in the controls. The surviving fractions and predicted  $LD_{50}$  derived from the recipient experiments are summarized in Table VII.

<sup>a</sup> The range is based on comparison of  $D_{37}$ 's for AET and control curves excluding and including, respectively, the "shoulder points".

Experiments were also conducted with CFU's from the femurs and spleens of endotoxin-treated mice to determine if the survival curves might have any different properties than CFU-survival curves from non-treated control mice. The basic design is as was described above for "recipient experiments", except that in the endotoxin groups the marrow and spleen CFU's were obtained from mice which had been injected with endotoxin 24 hours before sacrifice. The effect of endotoxin on CFU and nucleated cell content in these organs was described above in Table II. The results in Figure 9 show that endotoxin treatment did not alter the radiation sensitivity of CFU's derived from the spleen or femur.

The response of spleen CFU's, based on large and total nodule counts, is illustrated in Figure 10A. All observed points were considered in the fitted survival curves, since excluding points at < 75 or 150 R gave  $D_{37}$ 's which were within 1 R and extrapolation numbers within 0.02 units of the values derived by fitting all points. The control  $D_{37}$ 's for spleen CFU's from endotoxin-treated or control animals were essentially identical. Based on counts of large nodules, both  $D_{37}$ 's were 71 R, and based on total nodule counts, the  $L_{37}$ 's were 80 and 78 R, respectively. The only difference between the endotoxin and control curves was a slight displacement of the endotoxin curves on the X axis which produced small and statistically insignificant increases in the intercepts. Based on total counts the intercept was 1.38 compared with an intercept of 1.01 in controls; based on counts of large nodules, the increase was from 1.27 for control curve to 1.64 for the endotoxin curve. The possibility thus arises of some

small effect of endotoxin on the shoulder region of the survival curve.

On the other hand, this could result from endotoxin altering the size distribution of spleen nodules. This matter of size distribution will be considered in detail elsewhere.

The  $D_{37}$  of CFU's derived from the bone marrow of endotoxin-treated mice, 87 R, was only 5 R higher than the control value when all points were fitted (no point was below 100 R). The  $D_{37}$ 's and extrapolation numbers were the same based on either larger or total nodule counts. The intercept of 1.23 for the endotoxin curve was 0.52 units below the control intercept, but the difference was not significant. When the endotoxin curves were fitted to the points at 150 R and above, the extrapolation numbers and  $D_{37}$ 's were 1.46/84 R based on total counts and 1.40/85 R based on counts of large nodules.

Many sources of variation (dilution, injection and irradiation) are inherent to the recipient experiments as well as the other methods employed in the present study. Perhaps the greatest potential source of outright error is present in the recipient method when used in conjunction with protective procedures such as the injection of AET or endotoxin. At least three factors came into play which could increase the background number of nodules in the recipient spleen, and thus could significantly bias the data: 1) fractionation of exposures, early repair, and changes in sensitivity - recipient animals receive two exposures, a "primary" exposure and a "test" exposure (administered to the transplanted marrow or spleen CFU's) which are separated by ~2 hours. The process of early repair

could be initiated after the primary exposure, and this could result in an increase in the surviving number of CFU's in the recipient spleen.

This same effect could be produced by changes in the radiosensitivity of CFU's in the spleen which survive the primary exposure (31, 32);

2) CFU mobilization and migration to the spleen - earlier experiments showed that endotoxin causes an increased migration of CFU's to the spleen (19), and over a period of several hours AET could have the same effect. The results in Figure 1 showed that AET given after irradiation increased the number of endogenous spleen nodules. This curve in Figure 1 is clearly not a survival curve for CFU's indigenous to the spleen, but probably represents the effect of radiation on the ability of the spleen to sequester surviving CFU's which are mobilized into the circulation. Therefore, with the two-hour fractionation used in the recipient experiments, the lower the "primary" exposure, the more important this factor might be; 3) AET protection of the recipient spleen - mice receiving the primary exposure are injected with the test sample of marrow or spleen cells and are irradiated  $\sim$ 2 hours later in the presence of AET. The AET "protects" some fraction of the exogenous cells in the spleen at the time of irradiation, but in addition, the drug also protects CFU's in the spleen and in other sites in the recipient animals which have survived the primary exposure; the recipient animals extraspelnic CFU's, which should be protected by the AET, could contribute to the spleen nodule count via migration.

We have attempted to evaluate the composite effect of these factors on the CFU counts in the recipients' spleens. The results of control experiments on endogenous spleen nodules are summarized in Table VIII. First of all, most of the nodules observed in these animals given high exposures were quite small, < 0.5 mm. A single exposure of 900 R resulted in an average count of 1.0 nodule/spleen, based on total nodule count. This is higher than the expected value of  $\sim 0.3$  n/s based in Figure 1B, but is undoubtedly within the limits of sensitivity of the system. The two-hour fractionation (400 + 500 R) resulted in an increase in the total nodule count to 2.1 n/s. An increase would be expected on the basis of the considerations mentioned above. The total spleen nodule counts were also increased to 2.6 and 2.1 in animals receiving fractionated exposure with AET given before the second fraction. These results show that using the experimental design particular to the recipient-type experiments (Figures 6 - 10), the number of "background" nodules, especially small nodules, in the recipient's spleen is increased. However, the background of large nodules was not increased to a level which could confer significant bias. Since the background count of large nodules in the recipient's spleen was < 0.2 n/s, the exposure-response curves constructed from counts of large nodules are least subject to bias, and, based on the similarity of  $D_{37}$ 's based on counts of total and large nodules, it appears doubtful that even the total nodule counts were seriously biased.

## DISCUSSION

Whether an animal lives or dies may be predicted with some degree of reliability by evaluation of various functional indices of hematopoiesis. Yet, for many years the mammalian radiobiologist has sought more quantitative indices and has viewed with envy the highly quantitative data obtained from radiation studies with cell cultures in vitro. In 1961, Till and McCulloch described a means whereby the survival of hematopoietic stem cells could be evaluated in vivo in a fashion which would yield cell survival data that approximate cell culture methods in its quantitative aspects (1). The availability of this method and later development of another method of stem cell enumeration (33, 34) has given great impetus to studies designed to interpret radiation injury and recovery in the animal in terms of cell survival curves and population kinetics. In the present report we have extended our earlier studies concerning the relationship between CFU's and survival in the hopes of coming closer to grips with both the virtues and limitations of various methods of CFU enumeration in relation to their usefulness in predicting survival of animals. We felt that a comparison in which the effects of radioprotective procedures were evaluated by different methods of determining CFU survival would be of particular interest, since in our earlier endotoxin work different CFU methods yielded somewhat different answers concerning the effects of

endotoxin on the relative numbers of CFU's in the irradiated mouse (19, 24).

Based on the present results, we will first intercompare and discuss the properties of the CFU responses measured by the different methods, and then deal with the relationship between CFU's and the animal's survival.

We will initially consider endogenous spleen nodules in connection with  $D_{37}$ 's or slopes of the survival curves, the problem of size distribution(s) and the possibility of migration of CFU's to the spleen. In most of the earlier studies concerned with protective procedures, the endogenous method was used (13-15, 18-20, 22, 23). The present data indicate that dose-response studies with endogenous spleen nodules show a high degree of variability and may be quite sensitive to the nodule size criterion used for scoring. We are not aware that these points have been made before.

On the basis of our  $D_{37}$  of 66 R based on large nodules (0.5 mm+) and the value of 117 R based on total nodule counts (0.1 mm+), it appears that any  $D_{37}$  within this range could be generated by changing the size criterion.

If the group sizes were larger, it might be possible to show significant differences in  $D_{37}$  as a function of nodule size. The present estimate of 66 R is somewhat low compared with the often cited values of 70 - 100 R (7-9), but it is close to the value of 74 R derived from data of Smith et al. (16). On the other hand, it is somewhat higher than the 40 - 50 R values reported by Vacek and Sugahara (20). On the basis of the nodule size- $D_{37}$  relationships proposed here, it may be that the values cited by Vacek and Sugahara are related to their use of a large size criterion to facilitate nodule counting rather than real differences in the radiosensitivity.

of CFU's.

Using the endogenous method, we have studied the responses of spleen CFU's under conditions in which survival is increased by pre-irradiated administration of either bacterial endotoxin or AET. This permits evaluation of the relationship between survival of spleen CFU's and survival of the mouse. The administration of endotoxin or AET before irradiation significantly increased 30-day survival and increased the numbers of CFU's. Although in the treated animals CFU's were observed at much higher radiation doses than in control animals, the CFU survival curves were essentially parallel to the survival curves in the control animals. Neither endotoxin nor AET significantly changed the  $D_{37}$ . A change in the  $D_{37}$  for endotoxin was not expected based on our earlier work which showed no alteration in the radiosensitivity of CFU's in endotoxin-treated animals (14, 19), but a chemical protectant such as AET which does alter cellular sensitivity (25) might be expected to increase the  $D_{37}$ .

Using the closely related compound MEG, Smith et al. showed a significant change in the slope of the survival curve and concomitantly in the  $D_{37}$  (16). A significant increase in the  $D_{37}$  for endogenous CFU's in animals irradiated under hypoxic conditions has also been observed by Vacek and Suganara (20) and Phillips and Hanks (23). The protective drug cystamine was used by Juraskova and Tkadlecек (22) in studying survival of endogenous spleen nodules. The importance of changes in the slopes of the survival curves was less clear in their experiment, and changes in the  $D_{37}$  were not used to evaluate the drug's effectiveness.

In their experiments the numbers of spleen nodules were increased under conditions (injection 20 minutes before irradiation) which apparently did not change the slope of the CFU survival curve. However, at injection times closer to irradiation (< 8 minutes before the beginning or the mid-point of exposure) the slopes of the CFU-survival curves were quite shallow and the  $D_{37}$ 's would be high.

Our results with AET injected before irradiation appear to be more comparable to those of Juraskova and Tkadlecek with regard to the absence of a significant change in slope. The question therefore arises in connection with the various protective procedures as to why slope changes occur under certain circumstances and not under others. One factor could involve drug-induced mobilization of CFU's and their sequestration in the spleen. This was shown to occur following the administration of endotoxin (19). An AET-survival curve parallel to the control curve (resulting in no change to the  $D_{37}$ ) could occur as a composite of the following two-component curves: 1) the "expected" survival curves with an increase in the  $D_{37}$  and an intercept the same as the control; and 2) a curve describing the number of CFU's which, following drug injection, are redistributed and migrate to the spleen in animals receiving graded radiation exposures. Evidence for mobilization to the spleen following AET injection comes from the present experiments in which AET was given after irradiation. Under these conditions, the number of nodules occurring in the spleen was increased above the controls and was related to the radiation exposure.

which the animal sustained (Figure 1). Such a mobilization probably also occurs following the injection of colchicine, and Brecker *et al.* have suggested this may contribute to an increase in the spleen nodules when colchicine was given after irradiation (17). This redistribution might also partially explain the increased numbers of spleen nodules which occur following the injection of DNA, killed tumor cells, or milk (35). It may also be that differences in the size distributions of nodules between control and of treated animals could also influence the scoring and consequently the slope of the survival curve.

The value of the explanation offered here for parallel slopes must await more data and a more thorough analysis of the existing data. This explanation is not supported by the results of Smith *et al.* who showed a difference in the slope of the CFU-survival curve in MEG-treated mice (16). Even discounting their assumption of a common intercept<sup>a</sup>, a significant change in the  $D_{37}$  remains. Perhaps the important point is that when the endogenous spleen nodule method is used in connection with protective drugs, various pharmacological responses may impinge upon the test system, and the radiation-dose-response relationship might be aptly termed, as well as interpreted, as occurrence curves rather than true survival curves. Suffice it to say with the endogenous spleen nodule method the various factors which influence the slopes as well as the intercepts of the curves

<sup>a</sup> Under conditions of hypoxic protection or pre-treatment with cystamine, common intercepts are not observed with the endogenous method (22, 23).

are yet to be completely defined and resolved. Yet, in spite of the variability and the dearth of information about factors which impinge upon the system, the present data show that under certain conditions these curves can be used to predict the  $LD_{50}$  for endotoxin- or AET-treated animals within 5 - 10%. The matter of  $LD_{50}$  prediction will be considered to a greater extent below.

We will now consider the transplantation method of evaluating CFU's and discuss the effects of the protective agents on the relative numbers of CFU's in the femur and spleen both before and after irradiation; in addition, the  $D_{37}$ 's and extrapolation numbers derived by the various methods will be compared. Quantitative studies of CFU survival which are conducted by transplantation methods have several advantages over the endogenous method. By determining the relative number of CFU's in the femur and spleen both before and after irradiation, both the surviving number and the surviving fraction (or percentage) may be considered.

We first determined if the injection of either AET or endotoxin changed the number of CFU's present in the femur or spleen at the time the animals were to be irradiated. An injection of AET 15 minutes before sacrifice of the animal produced a small but statistically insignificant increase (11 - 19%)<sup>a</sup> in the CFU content of the spleen and femur; the nucleated cell content of these organs was unaltered. In contrast,

<sup>a</sup> This range is based on total and large nodule counts.

endotoxin given 24 hours before sacrifice produced significant increases of 21% in the nucleated cell content and 85 - 100%<sup>a</sup> in CFU content of the spleen. No change was observed in the CFU content of the femur, but the nucleated cell content declined by 40%. This resulted in an increase of 76 - 98%<sup>a</sup> in the number of CFU's/ $10^5$  nucleated cells in the femurs of endotoxin-treated animals. The present results showing an increased content of CFU's in the spleen of endotoxin-treated animals are at variance with the results of Smith *et al.* (36). The explanation for this difference is not known. The present results showing an increase in the ratio of CFU's/ $10^5$  nucleated marrow cells, but no net increase in the number of CFU's/femur are at variance with our report of an absolute increase in the CFU content of the femur (19, 24). In this case the difference may be ascribed to the use of only four donor mice in the earlier work.

A comparison of survival curves constructed from CFU data obtained by transplantation of marrow or spleen cells (donor and exogenous or recipient methods) differs from the endogenous survival curves in two important particulars, namely, AET increased the  $D_{50}$  for CFU's in both femur and spleen, and the  $\lambda$  rule size criterion did not materially influence the  $D_{50}$ . Moreover, the variability associated with donor method was greater than in the recipient. Neverthless, the 1 hour and 24 hour  $D_{50}$ 's, held similar  $D_{50}$  values of 10 and 15, respectively, for general CFU's in untreated animals. This agreement also existed with

<sup>a</sup> 50% increase in cell number and large colony counts.

regard to the shoulder region of these survival curves as derived from estimates of the extrapolation number or intercept value. Using the normal content of 9200 CFU's/femur (Table III) and the calculated Y intercepts of 15,400 or 17,300 (Figure 3, Table 5), an extrapolation number of  $1.68 - 1.88$  may be estimated from donor experiments. The comparable intercept derived from the exogenous studies was 1.74. In the presence of AET, the  $D_{37}$  derived by the donor method, 12<sup>3</sup> R, was quite close to the  $D_{37}$  of 12<sup>4</sup> R derived by the exogenous method. However, the Y intercept value of 1.28 derived from the donor experiments with AET was lower than the value of 1.74 in the recipient studies<sup>a</sup>. The AET-protection ratio (PR) estimated by the donor method,  $12^3/77 = 1.66$ , approximates the PR of  $143/82 = 1.75$ , estimated by the recipient method. Therefore, in most respects the responses described for femoral CFU's by the donor and recipient methods are in fairly close agreement.

The AET-protection ratios reported here for femoral CFU's may be compared with the results of earlier AET studies by Duplan and Fuhrer (21). Using the donor method to obtain three points on each survival curve, they obtained  $D_{37}$ 's somewhat higher than our's, 11<sup>3</sup> and 24<sup>3</sup> R, respectively, for femoral CFU's in control and AET-treated mice. Their survival curves also showed essentially a common intercept, and their protection ratio of

<sup>a</sup> Intercepts computed from the donor experiments are quite sensitive to errors in the estimates of normal CFU content in the femur or spleen (Table III), and we have no information on the effect of AET on transplantability of CFU's.

2.18 is ~30% higher than the value of 1.66 reported here. Some of the difference between their results and those reported here could relate to curve-fitting procedures, since we too obtained a protection ratio of 2.19 for spleen CFU's when points in the 190 - 280 R range were included in the regression (Table VI). We feel that there is uncertainty about the "shoulder width" and shape of the AET-survival curves shown in Figure 3, and if only points below 250 R fitted in the controls and points below 400 R were fitted in the AET group, the  $D_{37}$  estimates would be closer to those reported by Duplan and Fuhrer (21). This will be discussed in greater detail below.

A comparison of the radiation responses of spleen CFU's evaluated by the endogenous, donor, and recipient methods show some interesting differences as well as similarities. The control  $D_{37}$ 's obtained by the endogenous and exogenous methods were similar at 66 and 71 R, respectively, but based on the donor method, a somewhat lower value of 54 R was observed. In the presence of AET the  $D_{37}$  obtained by the donor method was 98 R and the estimate from the exogenous method was 124 R. Although the  $D_{37}$  values differ by 27%, the difference was not significant. The difference between the control  $D_{37}$ 's derived by these two methods was 31% (54 and 71 R), but in spite of these differences, the estimates of the AET-protection ratio were similar. The PR values are 97/54 = 1.80 for the donor experiments and 124/71 = 1.75 for the exogenous method.

Although AET significantly alters the radiosensitivity of CFU's in the marrow and spleen, the present results obtained by the recipient

method show that endotoxin does not share this effect. In these experiments marrow and spleen cells were obtained from endotoxin-treated mice, and the radiosensitivity of the resident CFU's was measured following transplantation into recipient animals. The  $D_{37}$  for spleen and marrow CFU's derived in this manner were within 5 R of the  $D_{37}$  values for the controls. Thus, it appears that endotoxin does not markedly influence the radiosensitivity of CFU's. This is consistent with our earlier observations based on endogenous spleen nodules (19). However, endotoxin-treated animals do show an earlier reappearance of circulating leukocytes (37-40) and CFU's in the femur and spleen (24, 36). The importance of these regenerative phenomena has been discussed elsewhere, and further comment concerning the numbers of CFU's surviving in the femur and spleen will be deferred until we present other survival results based on both donor and recipient methods, and data pertaining to CFU repopulation.

Before considering the relationship between the present data and radiosensitivity of the animal, we wish to mention the relationship between relative numbers of CFU's determined by most of the enumeration methods used here and the total numbers of CFU's which are actually present in the spleen and femur. Since no transplantation procedures are involved in measuring radiation responses of endogenous CFU's, it has been assumed that extrapolation of the survival curve to the Y intercept (0 exposure) yields an estimate of the total CFU population in the normal spleen (16). This procedure, however, does not consider the shoulder of the survival curve, and, thus, should overestimate the population. When transplantation

procedures are used to evaluate the total CFU content of the spleen, the "plating efficiency" or the percentage of injected CFU's which form spleen colonies must be considered. Siminovitch *et al.* (4) derived a factor of 17%, whereas, Playfair and Cole (41) reported that ~8% of marrow CFU's produced spleen colonies. Thus, with a known "recovery efficiency", one may calculate the absolute number of CFU's/organ from the relative number determined experimentally by transplantation procedures. Using this procedure, Smith *et al.* have reported "embarrassingly close" agreement between the estimates of ~34,000 for total spleen CFU content in normal animals which were derived from (1) the Y intercept of their survival curve for endogenous spleen nodules and from (2) the total number derived, using the factor of 17%, from transplantation of normal spleen cells into supralethally irradiated recipients (10). However, when the present data are treated in the same fashion, estimates of total spleen CFU content are obtained which differ by a factor of 1.1. Extrapolation of survival curves for endogenous nodules (Figure 1A) yields a Y intercept estimate of  $\sim 120^a$ ; whereas, an estimate of  $\sim 11$ , is derived from the transplantation data (Table II) utilising the factor of 17%. Only by assuming that 17% (relative number in normal spleen, 1.0) intercept value of 120 (from extrapolation of endogenous curve) of the injected CFU's produce spleen nodules can our two estimates be made to agree. The difference

<sup>a</sup> Based on responses of large nodules, this extrapolation procedure is not feasible for total nodule counts since the intercept value is ~1.

between the findings of Smith et al. and our own may be partially related to technical points such as the use of different mouse strains and the different size criterion for scoring. Also, the relative number of spleen CFU's is higher in their animals than in ours,  $\sim 5000$  vs.  $\sim 1800$ ; this could mean their recovery procedures are more efficient than ours. Moreover, in our hands the Y intercept values are rather variable, and we reported earlier (19) an intercept of 2000 which differ markedly, though not significantly, from the intercept reported here. Based on two sets of data reported by Smith et al. (12, 16), it appears that they too have observed similar variation in intercepts. Some of the differences observed within our results and those of Smith et al. may be related to the nodule size criteria. As mentioned above and to be described in detail elsewhere, both the  $D_{37}$  and the intercept value may be strongly influenced by the size criterion. In view of our uncertainties concerning intercept value and "recovery efficiency", we deem it inappropriate at this time to attempt to discuss the results of the present donor or exogenous experiments in terms of absolute numbers of CFU's/organ or CFU's/mouse.

The potential of the CFU as a tool whereby radiation lethality in the animals may be related to stem cell survival was fully discussed by Till and McCulloch in their early contributions (1, 3). More recently this relationship has been discussed in some detail by Bond et al. (7), Bond and Robinson (8), Patt and Lala (42), and Robinson (9). Several experimental studies have been conducted by others to evaluate the

relationship between CFU's and survival of mice. Since the age-dependent changes in LD<sub>50</sub> and CFU responses are not directly related to the present results, the ensuing discussion will deal principally with protectants.

In general, protection experiments in which endotoxin or colchicine were used to increase survival did not support a direct correlation between endogenous spleen nodules and survival. These substances, in effect, produced either too many nodules or produced an increase in nodules under circumstances in which survival was not increased (14, 15, 17-19). On the other hand, protection experiments by Smith *et al.* with MEG (16), Vacek and Sugahara and Phillips and Hanks with hypoxia (20,23), and Juraskova and Tkadlecek with cystamine (22), generally support the correlation between spleen nodules and survival. Smith *et al.* (16) reported that the protection ratio of 1.67 for endogenous CFU's was in close agreement with the PR of 1.59 which was based on mortality responses. Our results with endogenous nodules cannot be treated as were the data of Smith *et al.*, since AET-pretreatment produced no significant difference in D<sub>37</sub>. However, a protective ratio of some validity may be derived from these data by comparing the radiation exposures which result in the same number of nodules in the presence and absence, respectively, of AET. For example, if exposures producing 1.5 n/s were 423 in controls and 574 in AET-treated mice, the PR would be 574/423 = 1.36. This method of derivation was used by Juraskova and Tkadlecek and may be used with the present data, but a problem arises in that the PR is not independent of the number of nodules at which the comparison is made. This is attributable to small differences in slopes

of the survival curves. Based on a comparison of large nodules in control and AET-treated mice, the range of protection ratios is from 2.18 at 20 n/s to 1.82 at 0.5 n/s (from Figure 1A). Thus, in the readily countable range of 5 - 15 n/s, the PR's of 1.99 - 2.14 are somewhat higher than the PR for 30-day survival, but by extrapolation to higher radiation exposures where efficient counting is impossible due to the paucity of nodules, the PR approximates the PR of 1.81 for 30-day survival. This result is comparable to that obtained by attempting to predict the  $LD_{50}$  for AET on the basis of a 50% probability of death when the number of nodules in the spleens of AET-treated animals reaches the number expected at the  $LD_{50}$  for controls. Using this extrapolation procedure, the predicted  $LD_{50}$  was within 5% of the observed  $LD_{50}$  (Table I). At present, we can offer no clear explanation for the improved predictive values derived by extrapolation to high exposure range. At present, we feel this is a fortuitous property of the "occurrence curve" in AET-treated animals, and this property could result from factors such as mobilization of CFU's to the spleen and changes in nodule size distribution which are accompanied by changes in the slope of the curve. This matter will be considered in greater detail elsewhere.

The CFU-survival curves derived by the donor or recipient method are more readily interpretable in terms of their relationship to the  $LD_{50}$  of the animal. The earlier report of Duplan and Fuhrer (21), based on experiments of the donor type, generally supported the relationship between survival of femoral CFU's and survival of the animals, as they pointed

out that PR for CFU's of 2.2 was somewhat higher than the PR of 1.8 for 30-day survival. Their data were not treated in terms of surviving number of surviving fraction at the  $LD_{50}$ 's. As mentioned above, their observation of a rather high PR for marrow CFU's could be related to curve-fitting procedures. For example, in the present donor experiments with femur CFU's, PR's approaching 2.0 were obtained for both femoral and splenic CFU's when points above 74 and 149 R were fitted in the control and AET group; whereas, fitting the points above 149 and 287 R, respectively decreased the PR's to  $\sim 1.7$ . Most of the change in the PR's resulted from the decrease in  $D_{37}$  for AET-treated CFU's from 158 to 128 R. We wish to again call attention to the fact that in the donor experiments with AET, CFU survival in both the femur and spleen was lower than expected at exposures of  $< 250$  R. In addition to the survival curves shown in Figure 4, one could also fit curves with more shallow slopes to the points at exposures lower than 250, and thus generate two component curves for both the femur and spleen. If this were done, the intercepts of the AET curves would be lower than in the controls. Toxicity of AET or radiosensitization could produce this effect and might help explain some of our earlier recovery studies with mice given AET (26), but, at present, we can only state that the radiation responses in the lower exposure range are undefined. More points are being added to the AET curve in the donor experiment in the hope of clarifying the responses in the lower exposure range and obtaining better estimates of the  $D_{37}$ .

The results of the present donor and recipient experiments and the relationships between these results and the predicted  $LD_{50}$  for AET-treated mice are summarized in Tables VI and VII. The range of PR's for AET (1.66 - 1.80) is reasonably close to the PR of 1.31 for  $LD_{50}$  survival. This observation, plus the fact that the AET- $LD_{50}$  may be predicted within 2 - 12%, based on 50% probability of death when the surviving number (or fraction) in the AET-treated animals is reduced to the number surviving at the  $LD_{50}$  for controls, indicates that survival of CFU's and survival of the animals are highly correlated.

This might be interpreted to indicate that the CFU is the "stem cell", the sensitivity and numbers of which, determine the radiosensitivity of the animal. However, we feel that this is not necessarily the case, for there are other CFU data which do not support this positive correlation. We reported earlier that the extent of CFU repopulation in the femur was not positively correlated with the split-dose  $LD_{50}$  (24), and Sugahara et al. observed that under conditions of fractionated irradiation the number of CFU's does not correlate with the animals' radiosensitivity<sup>a</sup> (42). Thus, the data available at this time indicate that under certain conditions CFU responses and the animals' radiosensitivity are positively correlated, but under other conditions they are not.

<sup>a</sup> Personal communication.

We propose that the extent of the correlation depends on whether the "normal" steady-state relationships prevail between various marrow "compartments" or population components at the time of irradiation, or whether these relationships have been perturbed by some factor such as a previous recent exposure to irradiation. Perhaps in the regenerating marrow, when normal steady-state relationships have been altered and differentiation pressures which impinge on the stem cell compartment(s) and their progeny are changed, priority is assigned to proliferation of the compartment, the cells in which determine the radiosensitivity of the animal. This is accomplished at the expense of the allocation of cells to compartments in which proliferation and differentiation occur. This may only be a transient phenomenon, but its existence is supported by the fact that in split-dose experiments the animal's  $LD_{50}$  has returned to near normal at the time when CFU repopulation has just begun (24). Extension of this logic would require that the CFU be classified as a stem cell with pluripotent potential, but not as the stem cell which determines the radiosensitivity of an animal. This logic would further require the existence of (a) stem cell(s) more primitive than the CFU.

It is important to stress that the present results showing a high degree of correlation between CFU responses and the  $LD_{50}$  should not be taken to support the idea that the CFU is the stem cell which determines radiosensitivity. This correlation would be expected to exist as long as the relationship between others, perhaps more primitive, "stem cell(s)"

and the CFU were to remain constant. During marrow regeneration after irradiation, these relationships change, and CFU repopulation in the femur (24) appears to be most closely correlated with changes in the numbers of leukocytes which occur several days later (40). It should also be pointed out that during hematopoietic regeneration, CFU's may be very positively correlated with survival potential of the animal, as is the case with granulocytes (37), but as we have shown earlier, survival potential after a single exposure, and recovery from radiation injury as described by the return toward a normal LD<sub>50</sub> following a sublethal exposure, are capacities which vary quite independently (26, 43). These findings and the speculation offered here support the admonition of Patt to the effect that a great deal of biology exists between the "stem cell" and the survival of an animal (44).

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TABLE I

$LD_{50/30}$  for LAF<sub>1</sub> Female Mice Given AET or Endotoxin

Group	$LD_{50}(R)$	Slope of Exposure-Response Curve	No. Points	No. Animals
Controls <sup>a</sup>	721 (708-731)	21.0 (13.1-28.0)	8	165
AET <sup>b</sup> 15 min	1313	16.3	10	213
Pre	(1296-1328)	(11.2-21.2)		
AET 15 min	745	11.5	8	142
Post	(726-760)	(7.1-15.9)		
Endotoxin <sup>c</sup> 24 hrs	940 (928-954)	16.4 (11.4-21.3)	12	183
Pre				

<sup>a</sup> 95% confidence intervals in parentheses.

<sup>b</sup> 275 mg/kg given i.p.

<sup>c</sup> 5 mg given i.p.

TABLE II

$LD_{50}$  Estimate for AET- or Endotoxin-Treated Mice Derived by Extrapolation  
of "Survival Curves" for Endogenous Spleen Nodules

Experimental group	Observed $LD_{50}$ (R)		Expected $LD_{50}$ (R)		Difference from Expected $LD_{50}$		
	Large <sup>b</sup>	Total <sup>b</sup>	at 0.09 N/S		0.09 N/S (R)	%	at 1.2 N/S (R)
			at 0.09 N/S	at 1.2 N/S			
Controls	721	0.09	1.20	--	--	--	--
AET Before	1313	0.05	0.15	1255	1120	-58	5
AET After	741	0.50	1.60	895	780	+154	21
Endotoxin Before	111	1.20	2.56	1115	995	+196	21
						+76	9

a N/S indicates modules/spleen.

b The columns headed "Large" and "Total" refer to the nodule sizes criteria of 0.5+ (Figure 1A) and 0.1+ (Figure 1B), respectively.

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TABLE IV

Estimates of the numbers of CFU's surviving are based on the mean number of spleen colonies times the dilution and, thus, do not include injected CFU's which do not go to the spleen.

Exposure (R)	LIVER		Exposure (R)	SPLAEN	
	0.1+	0.5+		CFU Survival and Size Criterion	CFU Survival and Size Criterion
Concentrations Control	100	3140	5720	100	957
		(6034-1471)	(45-7-633)		(570-1344)
	120	4273	3144	100	834
		(3556-4539)	(2540-3740)		(749-1038)
	150	3763	2603	120	709
		(3211-4347)	(2321-3006)		(555-863)
	180	2303	1495	150	477
		(2370-3242)	(1602-2328)		(397-556)
	200	1693	1130	180	244
		(1403-2304)	(187-1533)		(169-320)
	240	1009	574	200	172
		(70-1231)	(393-754)		(125-219)
Apparatus Control	280	723	477	220	178
		(563-393)	(371-584)		(154-202)
	300	544	392	240	95
		(418-670)	(242-542)		(61-130)
	320	566	343	280	79
		(436-697)	(277-410)		(54-104)
	360	244	147	300	26
		(206-236)	(120-175)		(16-36)
	400	7676	5720	350	715
		(6004-1471)	(45-7-633)		(474-556)
	420	4640	3200	400	1179
		(415-5142)	(252-3500)		(1034-1324)
Apparatus Control	440	4917	2730	420	522
		(3830-5304)	(2342-3234)		(340-704)
	460	3245	2437	440	437
		(2751-3739)	(2073-2096)		(314-559)
	480	3160	2060	480	241
		(2780-3540)	(1753-2327)		(180-302)
	500	1722	1130	520	262
		(1513-1931)	(327-1333)		(198-326)
	520	1453	999	550	198
		(1322-1584)	(869-1090)		(172-224)
	540	616	434	580	153
		(501-731)	(359-569)		(124-181)
Apparatus Control	560	648	441	620	91
		(525-772)	(353-529)		(73-108)
	580	777	559	660	48
		(664-890)	(469-649)		(33-63)

TABLE V

Donor Experiments; The Influence of Curve-Fitting  
Procedures and Nodule Size Criteria on  $D_{37}$  for Intercept Values.

	Points Fitted	Size Criterion	Intercept $\times 10^3$	$D_{37}$	Correlation Coefficient <sup>a</sup>
CONTROLS	120+	0.1+	21.8	81(71-96)	0.991
	"	0.5+	15.4	80(65-103)	0.982
	150+	0.1+	27.2	76(66-90)	0.994
	"	0.5+	17.3	77(59-112)	0.976
<hr/>					
FEMUR	192+	0.1+	20.6	153(111-248)	0.949
	"	0.5+	13.1	158(118-240)	0.959
	238+	0.1+	35.6	127(77-370)	0.942
	"	0.5+	24.1	128(84-276)	0.960
<hr/>					
CONTROLS	120+	0.1+	5.2	60(48-79)	0.979
	"	0.5+	4.3	54(44-71)	0.980
	150+	0.1+	5.4	60(44-92)	0.969
	"	0.5+	4.7	54(40-82)	0.970
<hr/>					
SPLEEN	192+	0.1+	2.7	128(103-168)	0.960
	"	0.5+	2.0	118(95-153)	0.950
	238+	0.1+	3.3	117(113-249)	0.942
	"	0.5+	4.2	97(77-132)	0.940

a The correlation coefficient is an index of the goodness of fit of the regression line to the observed points. The closer the value is to unity, the better the fit.

TABLE VI

Interactions between the Predicted and Observed Mortalities  
in a Function of Curve-Fitting Procedures,  
Particle Size Distribution,  $\beta$ ,  $\alpha$ ,  $\beta_1$ ,  $\beta_2$

Points Fitted	Origin of CFU's	Treatment	$D_{50}$ (hr)	Relative ratio <sup>a</sup> of $D_{50}$ 's	Survival fraction ( $S_{50}$ ) at Measured $LD_{50}$	Relative Mortality of CFU's surviving at Measured $LD_{50}$	Predicted $LD_{50}$ (hr)	Difference from observed $LD_{50}$ (hr)
149+	Femur	Control	12 <sup>b</sup>	1.00	$3.65 \times 10^{-5}$	1.46	--	--
		AET	12 <sup>b</sup>	1.00	$3.65 \times 10^{-5}$	0.97	1246	-67
287+	Spleen	Control	54	1.00	$1.43 \times 10^{-6}$	$0.69 \times 10^{-3}$	--	--
		AET	97	1.00	$1.31 \times 10^{-5}$	$5.47 \times 10^{-3}$	1293	-26
149+	Femur	Control	80	2.00 <sup>c</sup>	$2.32 \times 10^{-4}$	1.34	--	--
		AET	150	2.00 <sup>c</sup>	$3.51 \times 10^{-4}$	3.25	1405	-92
287+	Spleen	Control	54	2.16	$1.16 \times 10^{-5}$	$7.60 \times 10^{-3}$	--	--
		AET	114	2.16	$1.41 \times 10^{-5}$	$2.74 \times 10^{-2}$	1475	+152

a The ratio of  $LD_{50}$ 's for AET-treated and control mice is 1512/1721 = 1.12.

b Based on the exposure required to reduce the surviving number or fraction of CFU's to the level observed at the  $LD_{50}$  for nontreated control mice.

TABLE VII

Protection Ratios, Surviving Fractions, and  $LD_{50}$  Predictions from Recipient Experiments.  
 Module Size Criterion 0.5 - 2.0 mm.

Origin of CFU's	Extrapolation Number/ $D_{37}$ (R)	Ratio <sup>a</sup> of $D_{37}$ 's	Surviving Fraction (S/S <sub>0</sub> ) at Measured $LD_{50}$	Relative Number of CFU's Surviving at Measured $LD_{50}$	Predicted <sup>b</sup> $LD_{50}$ (R)	Difference from Observed AET $LD_{50}$ of 1313 R R %
Perir	---	1.75/82	$2.45 \times 10^{-4}$	---	---	---
AET	1.74/143	1.75	$1.72 \times 10^{-4}$	---	1265	-48 4
Spleen	---	1.28/71	$4.58 \times 10^{-5}$	---	---	---
AET	1.35/124	1.75	$3.22 \times 10^{-5}$	---	1265	-48 4

a The ratio of  $LD_{50}$ 's for AET-treated and control mice is 1.82.

b The exposure required to reduce the S/S<sub>0</sub> to the level observed at the  $LD_{50}$  in nontreated controls.

TABLE VIII

**The Effect of Fractionation and AET  
on Numbers of Endogenous Spleen Colonies**

Exposures (R)	No. Spleens Counted	Mean Colony Count/Spleen	
		Total 0.1 mm+	Large 0.5 mm+
900	15	1.0 (0.45 - 1.55) <sup>a</sup>	0
400 + 500 <sup>b</sup>	29	2.1 (1.36 - 2.84)	0
400 + AET <sup>c</sup> 600 <sup>b</sup>	15	2.6 (1.94 - 3.25)	0.07
545 + AET 570 <sup>b</sup>	15	2.1 (1.39 - 2.74)	0.13

a 95% confidence interval.

b The interval between fractions was 2 hours.

c AET given ~15 minutes before irradiation.

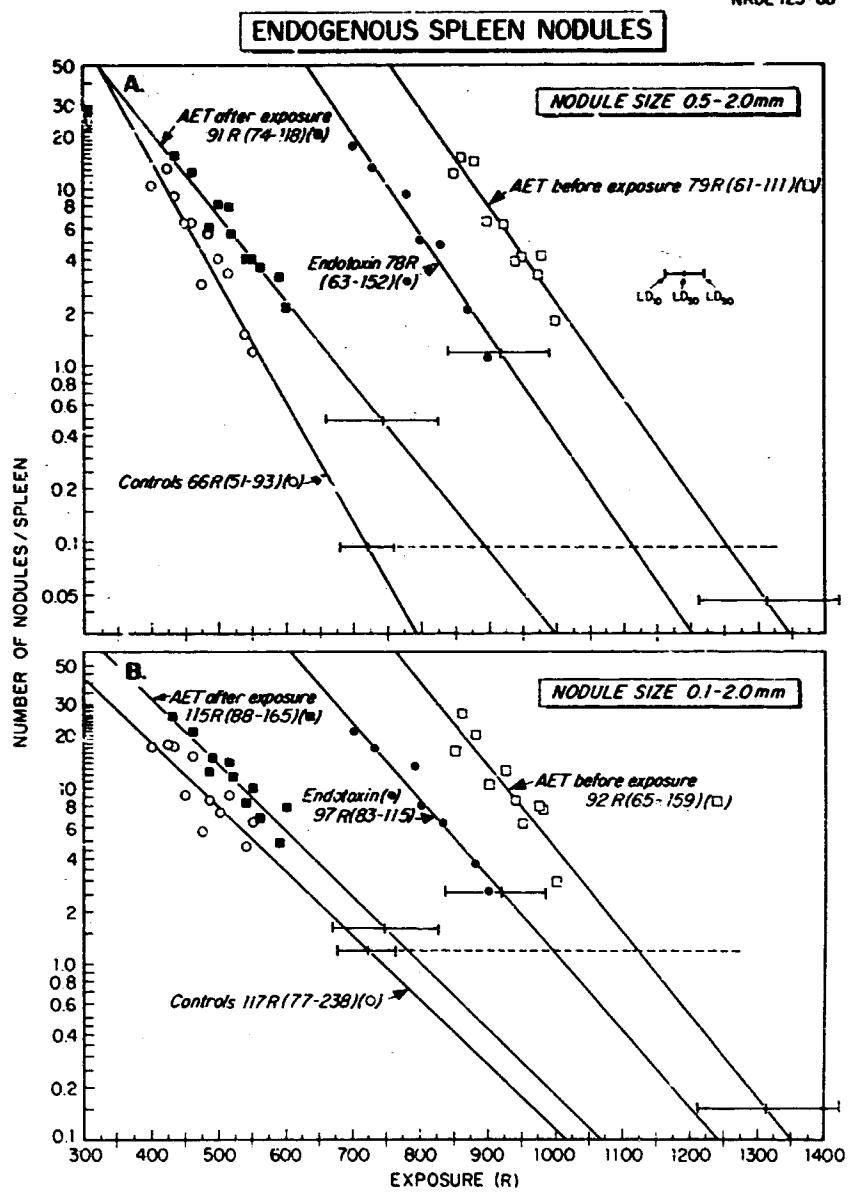


Figure 1. Relationship between radiation exposure and number of nodules/spleen. Each point represents the average mean of 11 - 15 spleens.

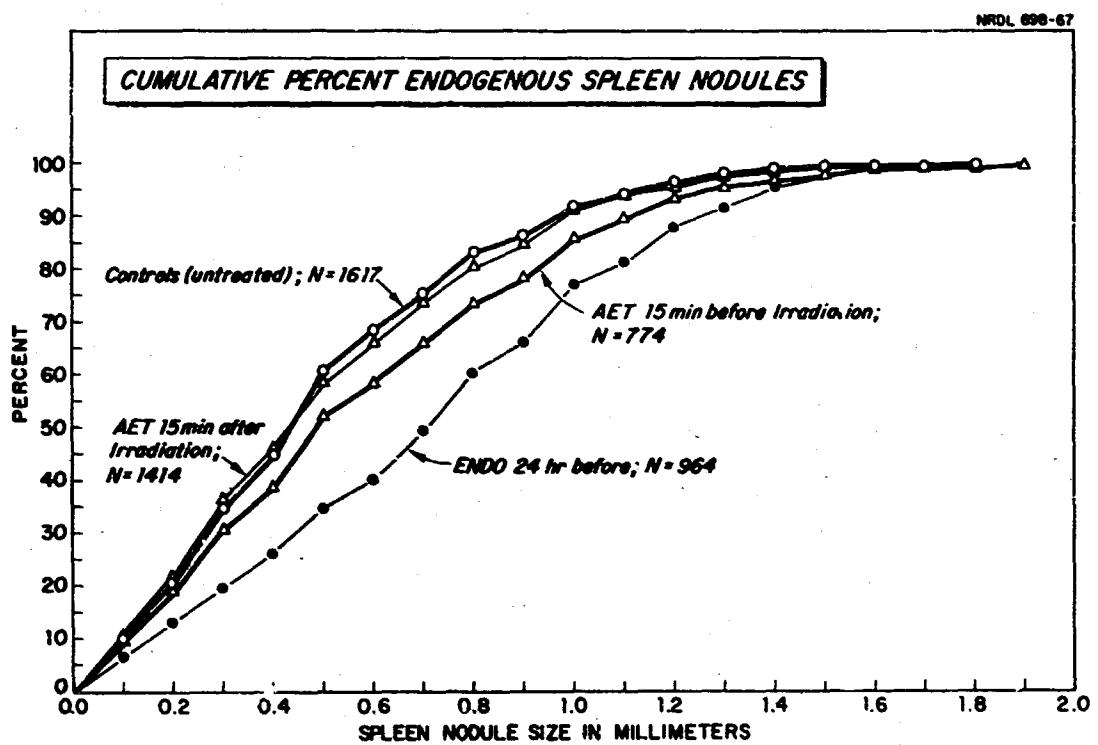


Figure 2. Size distribution of endogenous spleen nodules.

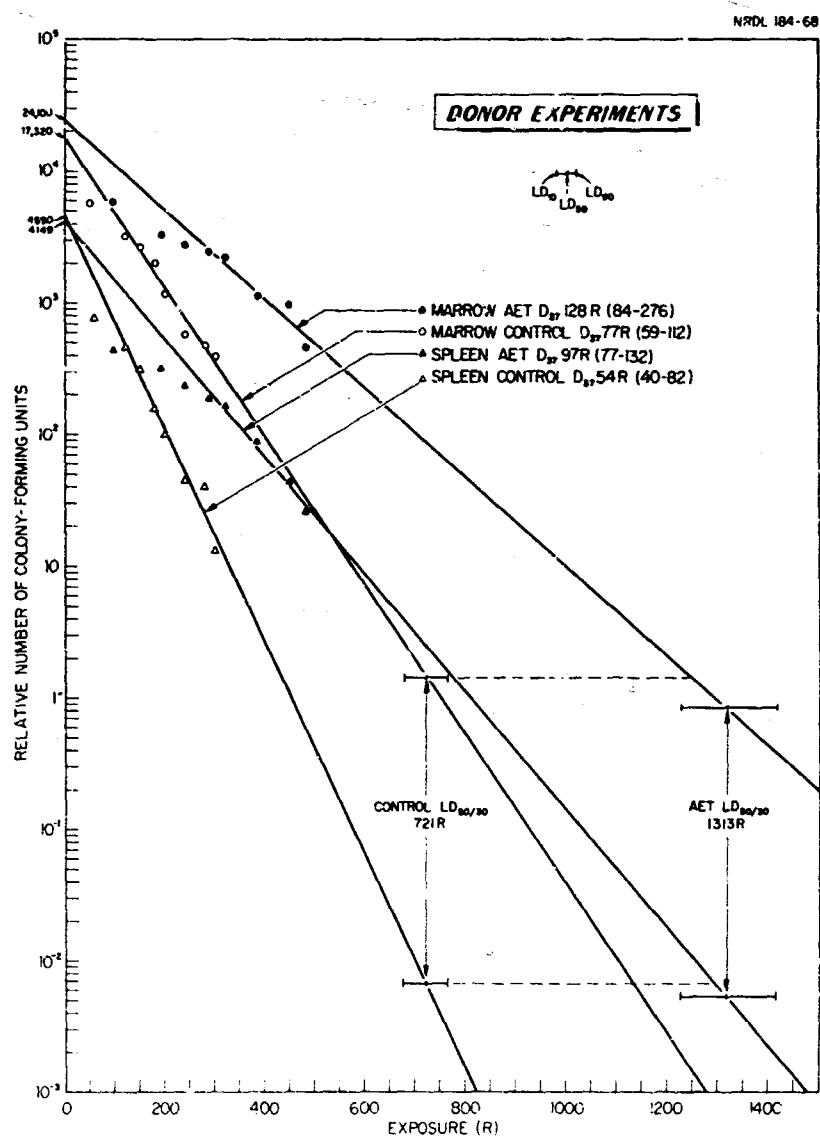


Figure 3. Radiation exposure-response relationship of CFU's in the spleens or femurs of controls or AET-treated mice. Each point represents the mean CFU count based on 11 - 15 spleens.

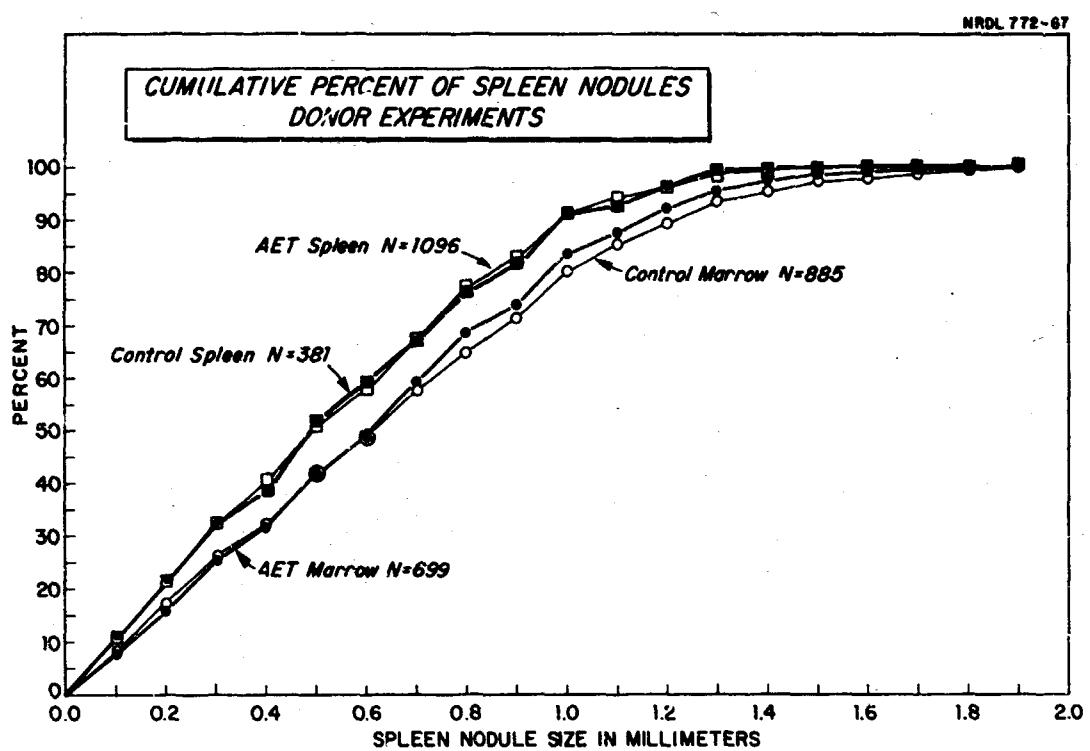


Figure 4. Size distribution of spleen nodules produced by CFU's derived from controls and mice treated with AET 15 minutes before irradiation. The number of nodules sized are indicated by N in the figure.

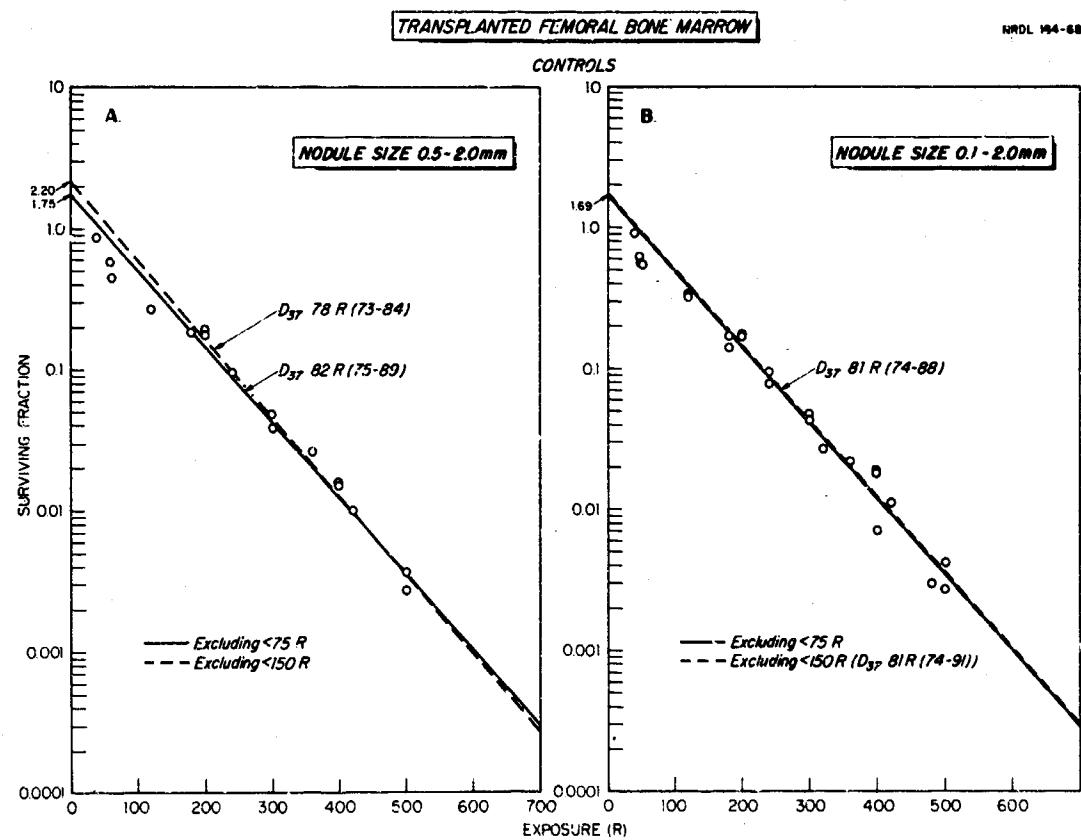


Figure 5. Survival curves for CFU's derived from normal femoral bone marrow. Each point represents the mean from counts of 11 - 15 spleens.

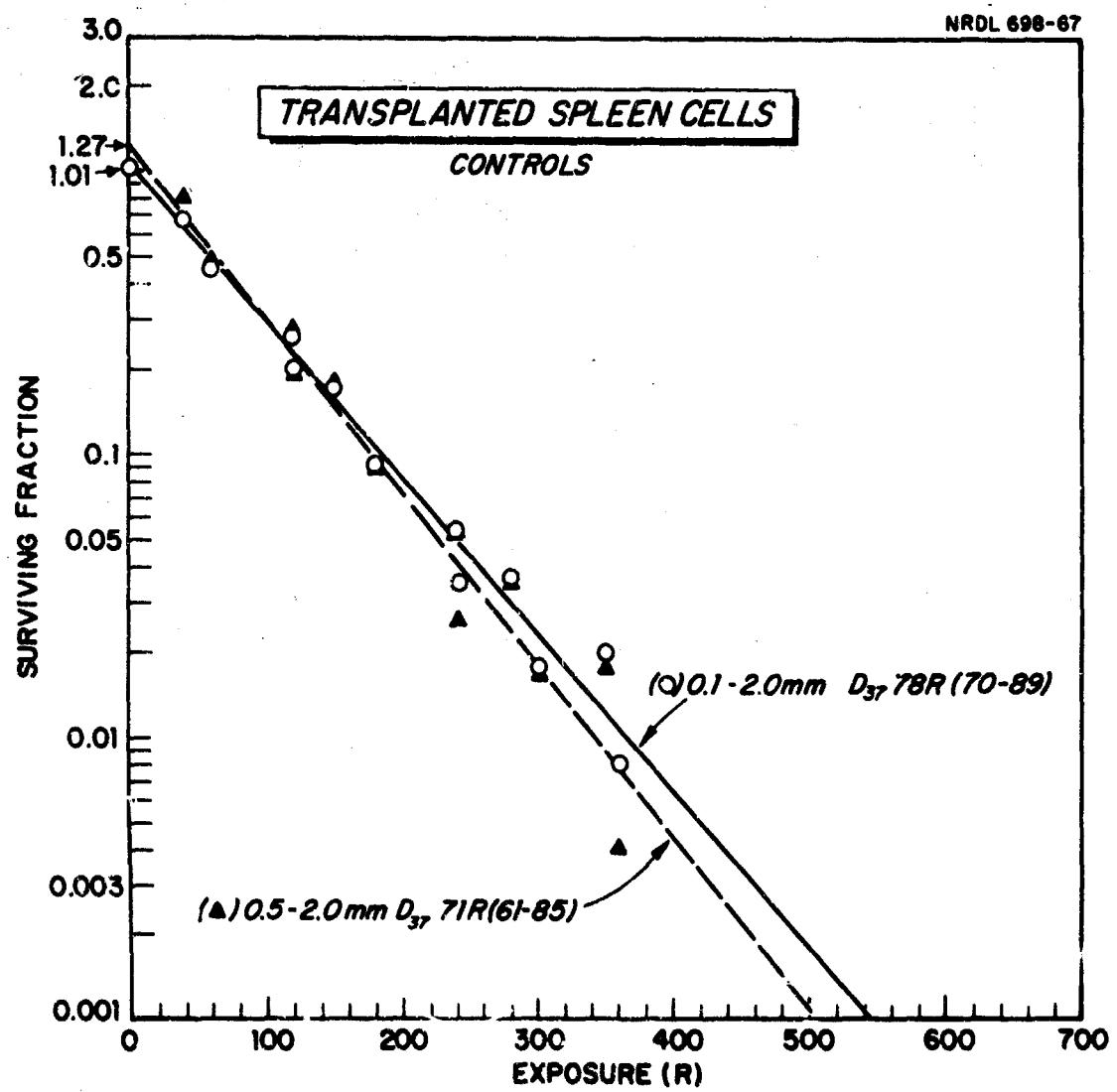


Figure 6. Survival curve for CFU's derived from normal spleens. Each point is based on nodule counts in 11 - 15 spleens.

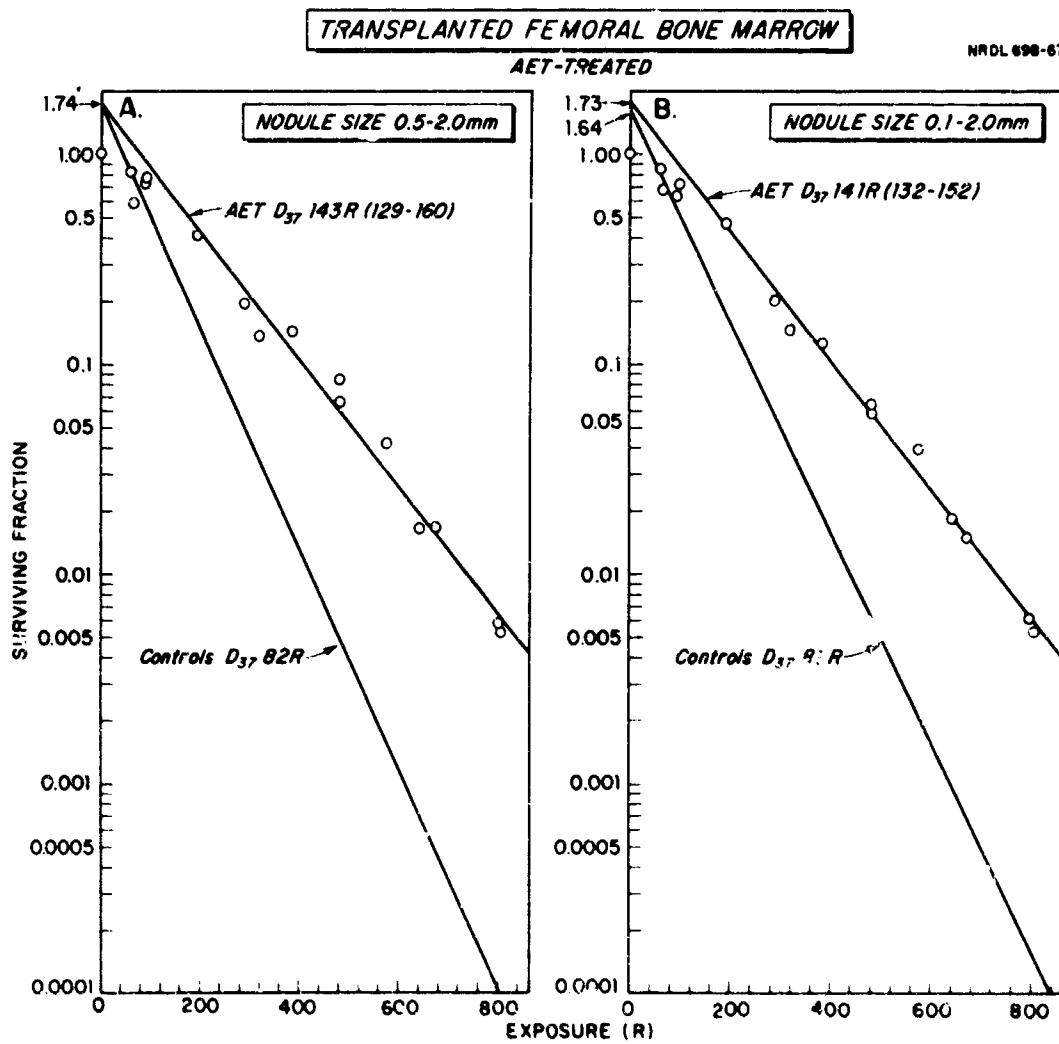


Figure 7. Survival curve for marrow CFU's irradiated in the presence of AET. The drug was given 5 - 15 minutes before exposure. Each point is based on the nodule counts in 11 - 15 spleens.

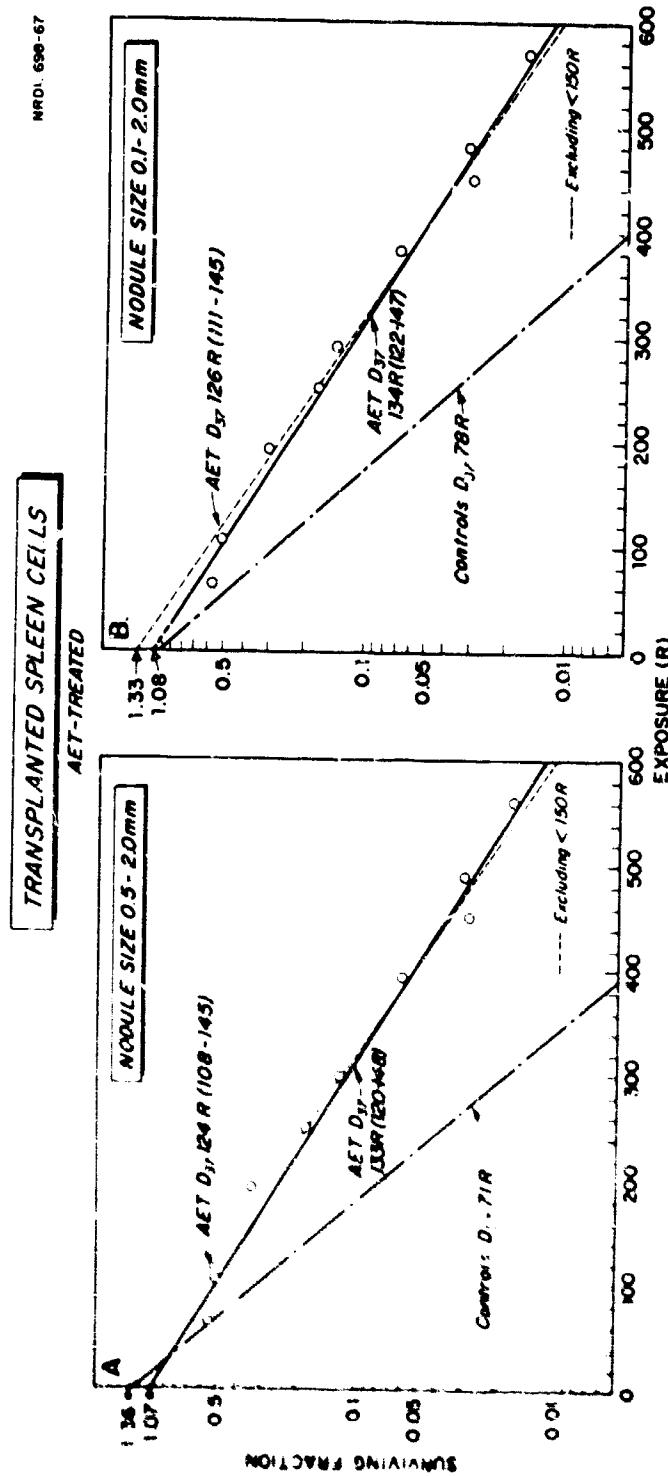


Figure 3. Survival curve for spleen CFU's irradiated in the presence of AET. The drug was administered 5 - 15 minutes before irradiation. Each point is based on nodule counts in 11 - 15 spleens. The solid line is fitted to all points and the broken line is fitted to points < 150 R. The irregular lines show the control curves from Figure 7.

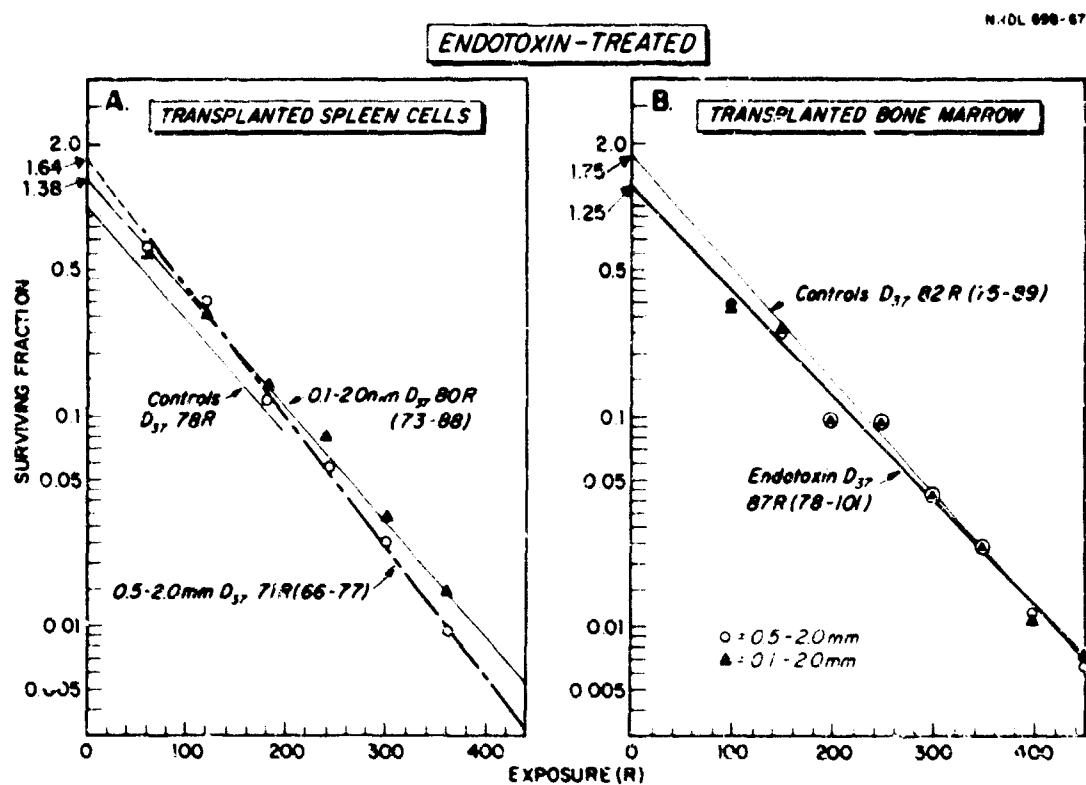


Figure 5. Survival curves for CFU's obtained from mice given 50  $\mu$ g of endotoxin 24 hours before sacrifice. Each point is based on counts of 11 - 15 spleens. Figure 5A shows the spleen CFU responses. The control curve shown without points has an intercept of 1.11 (from Figure 5) and is based on 1000 counts. The control curve for large cordiles (from Figure 5), which is not shown, has a  $D_{50}$  of 71 R and an intercept of 1.11. Figure 5B shows the marrow CFU responses. Control curves from Figures 5A and 5B are shown without points.

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13. ABSTRACT When mice are given radiation exposures producing the hematopoietic syndrome, it is assumed that it is the killing of hematopoietic stem cells and the leuko- and thrombocytopenias which ultimately develop that predispose the animals to infection, hemorrhage and death. The colony-forming unit (CFU) has many attributes of a (the) hematopoietic stem cell, and it might be expected that a high correlation should exist between CFU survival and survival of the animal. Some earlier studies have supported this correlation, whereas, others have not. In the present experiments three methods of CFU enumeration (endogenous, exogenous, and donor) have been used to evaluate this correlation in mice "protected" with AET or bacterial endotoxin. The results show that the different CFU enumeration procedures yield somewhat dif- ferent results, yet under certain conditions the LD <sub>50</sub> 's for AET- or endotoxin- treated mice may be predicted within 5 - 10% from CFU survival curves. In spite of the good correlation between CFU survival and probability of survival of the mouse, it is proposed that the CFU is not the stem cell which determines the radiation sensitivity of the mouse.		

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